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(54) Title: FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF					
(57) Abstract					
The present invention is directed to novel fluorescent proteins from non-bioluminescent organisms from the Class Anthozoa. Also disclosed are methods of identifying nucleic acid sequence encoding the fluorescent proteins and further analyzing the proteins.					

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**FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES
OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND
USES THEREOF**

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BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of molecular biology. More specifically, this invention relates to novel fluorescent proteins, 15 methods of identifying the DNA sequences encoding the proteins and uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for 20 marking a protein, cell, or organism of interest. Traditionally, a protein of interest is purified, then covalently conjugated to a fluorophore derivative. For *in vivo* studies, the protein-dye complex is then inserted into cells of interest using micropipetting or a method of reversible permeabilization. The dye attachment and insertion steps, 25 however, make the process laborious and difficult to control. An alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this method of protein labeling include β -galactosidase, firefly luciferase

and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

A marker that does not require an exogenous cofactor or 5 substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

10 Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 15 635-642, discuss the use of wild-type GFP as a tool for visualizing subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by 20 Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel 25 (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets in a compact structure, where, in the center, an alpha helix containing the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in

general. However, the stability of GFP makes it sub-optimal for determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great utility of GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an

intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of 5 the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

10 **Figure 1** shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers).

15 **Figure 2A** shows multiple alignment of novel fluorescent proteins. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP). Two proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by 20 dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming beta-sheets are underlined; the residues whose side chains form the interior of the beta-can are shaded (according to Yang et al., *Nature Biotechnol.* 14, 1246-1251 (1996)).

25 **Figure 2B** shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

Figure 3 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia majano*, amFP486.

Figure 4 shows the excitation and emission spectrum of the novel fluorescent protein from *Clavularia*, cFP484.

Figure 5 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP506.

5 **Figure 6** shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP538.

Figure 7 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma striata*, dsFP483.

10 **Figure 8** shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, drFP583.

Figure 9 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia sulcata*, asFP600.

Figure 10 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dgFP512.

15 **Figure 11** shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dmFP592.

DETAILED DESCRIPTION OF THE INVENTION

20 As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

25 As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al., *Nature* 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for

expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3'

(carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination 5 sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

10 The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that 15 provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining 20 the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a 25 transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by 5 exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to 10 eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter 15 cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an 20 identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, 25 heterologous DNA includes coding sequence in a construct where portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

5 The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter code (A: alanine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophane; Y: tyrosine; X: any residue). NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552-15 59 is used.

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

20 In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence encodes a peptide having a sequence selected from the group 25 consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a

fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid 5 sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a 10 fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the 15 nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an 20 intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1Biological Material

5 Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

10

TABLE 1Anthozoa Species Used in This Study

Species	Area of Origination	Fluorescent Color
<i>Anemonia majano</i>	Western Pacific	bright green tentacle tips
<i>Clavularia</i> sp.	Western Pacific	bright green tentacles and oral disk
<i>Zoanthus</i> sp.	Western Pacific	green-yellow tentacles and oral disk
<i>Discosoma</i> sp. "red"	Western Pacific	orange-red spots oral disk
<i>Discosoma</i> sp. <i>striata</i>	Western Pacific	blue-green stripes on oral disk
<i>Discosoma</i> sp. "magenta"	Western Pacific	faintly purple oral disk
<i>Discosoma</i> sp.	Western Pacific	green spots on oral disk

“green”		
Anemonia sulcata	Mediterranean	purple tentacle tips

EXAMPLE 2**cDNA Preparation**

Total RNA was isolated from the species of interest according to the protocol of Chomczynski and Sacchi (Chomczynski P., et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was synthetized starting with 1-3 µg of total RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to the provided protocol with the only alteration being that the "cDNA synthesis primer" provided in the kit was replaced by the primer TN3 (5'- CGCAGTCGACCG(T)₁₃, SEQ ID No. 1) (Table 2). Amplified cDNA samples were then prepared as described in the protocol provided except the two primers used for PCR were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2) (Table 2) and the TN3 primer (Table 2), both in 0.1 µM concentration. Twenty to twenty-five PCR cycles were performed to amplify a cDNA sample. The amplified cDNA was diluted 20-fold in water and 1 µl of this dilution was used in subsequent procedures.

TABLE 2Oligos Used in cDNA Synthesis and RACE

5 TN3: 5'-CGCAGTCGACCG(T)₁₃
(SEQ ID No. 1)

T7-TN3: 5'-GTAATACGACTCACTATAGGGCCGCAGTCGACCG(T)₁₃
(SEQ ID No. 17)

10 TS-primer: 5'-AAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 2)

15 T7-TS:
5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 18)

20 T7: 5'-GTAATACGACTCACTATAGGGC
(SEQ ID No. 19)

25 TS-oligo 5'-AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG
(SEQ ID No. 53)

EXAMPLE 3**Oligo Design**

To isolate fragments of novel fluorescent protein cDNAs, 5 PCR using degenerate primers was performed. Degenerate primers were designed to match the sequence of the mRNAs in regions that were predicted to be the most invariant in the family of fluorescent proteins. Four such stretches were chosen (Table 3) and variants of degenerate primers were designed. All such primers were directed to 10 the 3'-end of mRNA. All oligos were gel-purified before use. Table 2 shows the oligos used in cDNA synthesis and RACE.

TABLE 3

Key Amino Acid Stretches and Corresponding Degenerate Primers Used for Isolation of Fluorescent Proteins

5

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVN ^G H (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5)	GE ^G a: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6)
	GEGNG (SEQ ID No. 8)	GE ^G b: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7)
		GN ^G a: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9)
		GN ^G b: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11)	NFP: 5' TTC CA(C,T) GGT
	GVNFP (SEQ ID No. 12)	(G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15)
		PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

EXAMPLE 4**Isolation of 3'-cDNA Fragments of nEPs**

The modified strategy of 3'-RACE was used to isolate the target fragments (see Figure 1). The RACE strategy involved two consecutive PCR steps. The first PCR step involved a first degenerate primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3' portion identical to the TN3 primer used for cDNA synthesis (for sequence of T7-TN3, Table 2). The reason for substituting the longer T7-TN3 primer in this PCR step was that background amplification which occurred when using the shorter TN3 primer was suppressed effectively, particularly when the T7-TN3 primer was used at a low concentration (0.1 μ M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-9002). The second PCR step involved the TN3 primer (SEQ ID No. 1, Table 2) and a second degenerate primer (Table 4).

TABLE 4

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
<i>Anemonia majano</i>	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
<i>Clavularia</i> sp.	NGH (SEQ ID No. 4)	GEGA (SEQ ID No. 6)
<i>Zoanthus</i> sp.	NGH (SEQ ID No. 4)	GEGA (SEQ ID No. 6)
<i>Discosoma</i> sp. "red"	NGH (SEQ ID No. 4)	GEGA (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
<i>Discosoma striata</i>	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
<i>Anemonia sulcata</i>	NGH (SEQ ID No. 4)	GEGA (SEQ ID No. 6) or NFP (SEQ ID No. 13)

5

The first PCR reaction was performed as follows: 1 μ l of 20-fold dilution of the amplified cDNA sample was added into the reaction mixture containing 1X Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH), 200 μ M dNTPs, 0.3 μ M of first degenerate

primer (Table 4) and 0.1 μ M of T7-TN3 (SEQ ID No. 17) primer in a total volume of 20 μ l. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1 μ l of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200 μ M dNTPs, 0.3 μ M of the second degenerate primer (Table 4) and 0.1 μ M of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10 sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

EXAMPLE 5Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel fluorescent protein cDNAs, two nested 5'-directed primers were synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were then amplified using two consecutive PCRs. In the next PCR reaction, the novel approach of "step-out PCR" was used to suppress background amplification. The step-out reaction mixture contained 1x Advantage 5 KlenTaq Polymerase Mix using buffer provided by the manufacturer (CLONTECH), 200 μ M dNTPs, 0.2 μ M of the first gene-specific primer (see Table 5), 0.02 μ M of the T7-TS primer (SEQ ID No. 18), 0.1 μ M of T7 primer (SEQ ID No. 19) and 1 μ l of the 20-fold dilution of the amplified cDNA sample in a total volume of 20 μ l. The cycling profile 10 was (Hybaid OmniGene Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of amplification was diluted 50-fold in water and one μ l of this dilution was added to the second (nested) PCR. The reaction contained 1X Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH), 15 200 μ M dNTPs, 0.2 μ M of the second gene-specific primer and 0.1 μ M of TS primer (SEQ ID No. 2) in a total volume of 20 μ l. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 12 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of amplification was then cloned into pAtlas vector (CLONTECH) according 20 to the manufacturer's protocol.

TABLE 5

Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC _ GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'- GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAAC (SEQ ID No. 28)	5'-CTGCACAAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTCCTGC (SEQ ID No. 30)	5'-TATCTTCATTCCT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTT (SEQ ID No. 34)	5'-ATTATCTCAGTGGAA TGGTTC (SEQ ID No. 35)

EXAMPLE 6**Expression of nFPs in *E.coli***

5 To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table 10 6). Both primers had 5'-heels coding_ for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading frames of the vector-encoded 6xHis-tag and nFP. The PCR was performed as follows: 1 μ l of 15 the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200 μ M dNTPs, 0.2 μ M of upstream primer and 0.2 μ M of downstream primer, in a final total volume of 20 μ l. The cycling profile was (Hybaid OmniGene 20 Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction endonucleases corresponding to the primers' sequence according to standard 25 protocols.

All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium (supplemented with 100 μ g/ml of ampicillin) at 37°C overnight. 100 μ l

of the overnight culture was transferred into 200 ml of fresh LB medium containing 100 μ g/ml of ampicillin and grown at 37°C, 200 rpm up to OD₆₀₀ 0.6-0.7. 1 mM IPTG was then added to the culture and incubation was allowed to proceed at 37°C for another 16 hours. The 5 cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

TABLE 6

Primers Used to Obtain Full Coding Region of nFPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' - <u>acatggatccgc</u> tcttcaaaca agtttatc (SEQ ID No. 36) BamHI	5' - <u>tagtactcgagcttattcgta</u> tttcagtgaaatc (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5' - <u>acatggatccaa</u> catttttga gaaacg (SEQ ID No. 38) BamHI S: 5' - <u>acatggatccaa</u> aggctctaacc accatg (SEQ ID No. 39) BamHI	5' - <u>tagtactcgagcaacacaa</u> accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5' - <u>acatggatccgc</u> tcaag cacgg (SEQ ID No. 41) BamHI	5' - <u>tagtactcgagggttggaa</u> ctacat tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5' - <u>acatggatcc</u> caggcttccaagaat gttatac (SEQ ID No. 43) BamHI	5' - <u>tagtactcgaggaggcca</u> aggtc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5' - <u>acatggatcc</u> cagggttccaagagtgt (SEQ ID No. 45) BamHI	5' - <u>tagcgagcttatcatgc</u> ctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5' - <u>acatggatccgc</u> tccctttaaagaagact (SEQ ID No. 47) BamHI	5' - <u>tagtactcgagtc</u> ttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5' - <u>acatggatcc</u> cagggttccaagaatgtat (SEQ ID No. 49) BamHI	5' - <u>tagtactcgaggcc</u> attacg ctaatac (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5' - <u>acatggatcc</u> cagggtgcactaaagaagaaatg (SEQ ID No. 51)	5' - <u>tagtactcgagattcg</u> gtttaat gccttg (SEQ ID No. 52)

EXAMPLE 7**Novel Fluorescent Proteins and cDNAs Encoding the Proteins**

Seven cDNA full-length cDNAs encoding fluorescent proteins were obtained (SEQ ID Nos. 45-51), and seven novel fluorescent proteins were produced (SEQ ID Nos. 53-59). The spectral properties of the isolated novel fluorescent proteins are shown in Table 7, and the emission and excitation spectra for the novel proteins are shown in Figures 3-11.

TABLE 7

Spectral Properties of the Isolated NEPs.

Species	NFP Name	Abs. Max. n m	Emission Maximum n m	Maximum Extinction Coeff.	Relative Quantum Yield*	Relative Brightness **
Anemonia majano	amFP486	458	486	40,000	0.3	0.43
Clavularia sp.	cFP484	456	484	35,300	0.6	0.77
Zoanthus sp.	zFP506	496	506	35,600	0.79	1.02
Zoanthus sp.	zFP538	528	538	20,200	0.52	0.38
Discosoma sp. "red"	drFP583	558	583	22,500	0.29	0.24
Discosoma striata	dsFP483	443	483	23,900	0.57	0.50
Anemonia sulcata	asFP600	572	596	56,200	<0.001	-
Discosoma sp. "green"	dgFP512	502	512	20,360	0.3	0.21
Discosoma sp. "magenta"	dmFP592	573	593	21,800	0.11	0.09

5 *relative quantum yield was determined as compared to the quantum yield of *A. victoria* GFP.

**relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

Multiple alignment of fluorescent proteins is shown in Figure 2A. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP, SEQ ID No. 54). The amino acid sequences of the novel fluorescent proteins are labeled as SEQ ID Nos. 55-63. Two 5 proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming β -sheets are underlined; the residues whose side 10 chains form the interior of the β -can are shaded. Figure 2B shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

The following references were cited herein.

1. Ormo et al., (1996) Science 273: 1392-1395.
- 15 2. Yang, F., et al., (1996) Nature Biotech 14: 1246-1251.
3. Cormack, et al., (1996) Gene 173, 33-38.
4. Haas, et al., (1996) Current Biology 6, 315-324.
5. Yang, et al., (1996) Nucleic Acids Research 24, 4592-4593.
6. Ghoda, et al., (1990) J. Biol. Chem. 265: 11823-11826.
- 20 7. Prasher D.C. et al. (1992) Gene 111:229-33.
8. Kain et al. (1995) Biotechniques 19(4):650-55.
9. Chomczynski P., et al., (1987) Anal. Biochem. 162, 156-159.
10. Frohman et al., (1998) PNAS USA, 85, 8998-9002.

Any patents or publications mentioned in this specification 25 are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, 5 molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope 10 of the claims.

WHAT IS CLAIMED IS:

1. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

5 screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

10

2. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence hybridizes to a primer 15 selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

3. A method of analyzing a fluorescent protein in a cell, 20 comprising the steps of:

a) expressing a nucleic acid sequence encoding a fluorescent protein in said cell, wherein said protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63; and

25 b) measuring a fluorescence signal from said protein.

4. The method of claim 3, further comprising the step of:

sorting said cell according to said signal.

5. The method of claim 4, wherein said step of sorting comprises sorting said cell by fluorescence activated cell sorting.

5 6. The method of claim 3, wherein said nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to said fluorescent protein, wherein said protein of interest is distinct from said fluorescent protein.

10 7. The method of claim 6, wherein the fluorescence signal indicates a presence of said gene of interest in said cell.

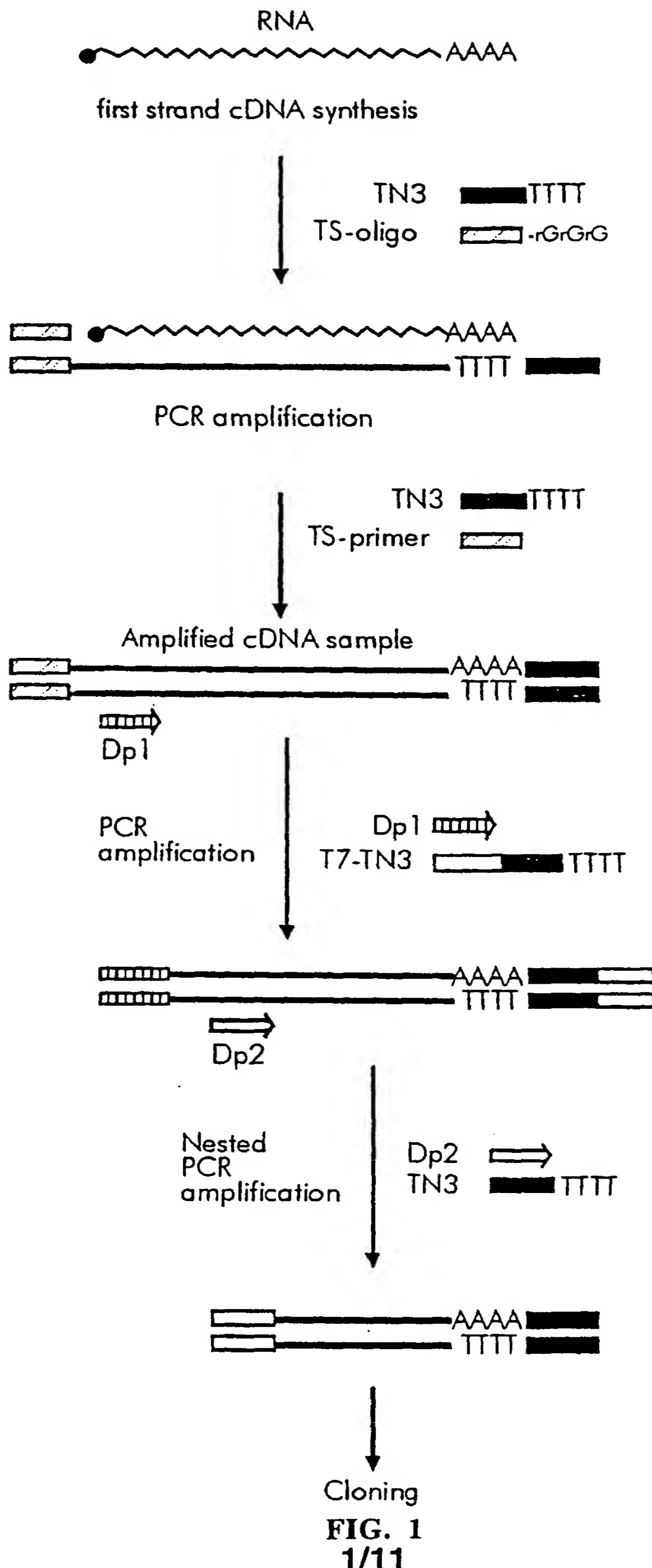
8. The method of claim 7, wherein said cell further comprises a protein of interest fused to said fluorescent protein.

15

9. The method of claim 8, further comprising the step of:

identifying an intracellular location of said fluorescent protein, thereby identifying an intracellular location of said protein of
20 interest.

10. An isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.



10	20	30	40	50	SEQ ID#
MSKGEELFTG. <u>VVPILVELGDVNNGHKFSVSGEGEGDATYGKLT</u> KFICTT. GKL PVP.. W GFP					54
MAQSKHGLTK. EMTMKYRMEGCVGDGHKFVITGEIGIGYPFKGKQAINLCVV.. EGGPLPFAE zFP506					57
--H----KE. -----H----N-----T----I..-----S- zFP538					58
MSWSKSVIKE. EMLIDLHLEGTFNHYFEIKGKGKGKPNEGTNTVTLEVT.. KGGPLPFGW dsFP483					59
. . . M-AL--. Y-K-N-TM--VV--LP-K-R-D----YQ-SQEL--T-V.. -----SY dgFP512					62
-RS--N----. F-RFKVRM---V--E--E-E-R-Y--H--K-K--. -----A- drFP583					60
M-C--N----. F-RFKVRM---V--E----E-E-R-Y--HCS-K-M--. -----AF dmFP592					63
. . . MASFLKK. TMPFKTTIEGTVNNGHYFKCTGKGEGNPFEGTQEMKIEVI.. EGGPLPFAF asFP600					61
MALSNKFIGD. DMKMTYIIMDGCVNNGHYFTVKGEENNGKPYEGTQTSTFKVTMANGGPLAFSE amFP486					55
KALTTMGVIKPDMKIKLKMEGNVNGHAFVIEGEGEKGPKYDGTHLNLEVKMAEGAPLPFSY cFP484					56
60	70	80	90	100	110
PTLVTTFSYGVQCFSRYPDHMKQHDFFKSAM.. :PEGYVQERTIFFKDDGNYKTRAEVKFEGD..					GFP
DILSAAFNFGNRVFTEYPQDIV.. DYFKNSC... PAGYTWDRSFLFEDGAVCICNADITVSVEEN					zFP506
-----G-K--D-I----- . . . -----G-----V-----K--					zFP538
HILCPQFQYGNKAFVHPDDIP.. DYLKLSF.. PEGYTWERSMHFEDGGLCCITNDISLTGN..					dsFP483
D--TTM----R--NY-E---. -IF-QTCSGPNG--S-Q-T-TY---V-TA-SN--VV-D..					dgFP512
D--S-----S-VY-K--A----. --K----. . . --FK---V-N----VVTV-Q-S--QDG..					drFP583
D--S-----S-VY-K--A----. --K----. . . --EK---V-N----VVTVSQ-S--KDG..					dmFP592
HILSTSCMYGSKTFIKYVSGIP.. DYFKQSF.. PEGFTWERTTYEDGGFLTAHQDTSLDGD..					asFP600
DILSTVFKYGNRCFTAYPTSM.. DYFKQAF.. PDGMSYERTFTYEDGGVATASWEISLKG..					amFP486
DILSNAEQYGNRALTKYPDDIA.. DYFKQSE.. PEGYSWERTMTFEDKGIVVKVSDISMEED..					cFP484
120	130	140	150	160	170
<u>TLVNRIELKGIDFEDGNILGHKLEYNNNSHNVYIMADKQKNGIKVNFKIRHNIEUDGSVQL</u>					GFP
CMYHESKEYGVNFPADGPVM. KKMTDNWEPSCERKIIIPVKQGILKGDVSMYIL. KDGGRJ.R					zFP506
-I--K-I-N-M-----T---A---M-----Y-					zFP538
CNYDIKFTGLNFPNGPVV. QKTTGWEPESTERLYP.. RDGVLIGDIHHALTVEGGGHYV					dsFP483
T----H-M-A--LD--MM--R-MK----IMFE ---L-R-D-AMS-LLK----R					dgFP512
--I-KV--I-V--SD--M. ---M---A----- . . . --K-E--K--KLKD---L					drFP583
--I-EV--I-V--SD--M. -RR-R---S-----K---M--RL----L					dmFP592
CLVYKVILGNNFPADGPVM. QNKAGRWEPAATEIVYE.. VDGVLRGQSLMALKCPGGRHLT					asFP600
CFEHKSTFHGVNFPADGPVM. AKTTGWDPSFEKMTV.. CDGILKGDVTAFLMLQGGGNYR					amFP486
SFIYEIRFDGMNFPNGPVM. QKTLKWEPESTEIMYV.. RDGVLVGDISHSLLEGGGHYR					cFP484
180	190	200	210	220	230
<u>ADHYQONTPIGDG. PVLLPDNHYLSTOSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK</u>					GFP
CQFDTVYKAKSV.. PRKMPDWHEIQHKLTREDRSDAKNQKWHLTEHAIASGALP					zFP506
-----S---E-----L-----Q-----FP---A					zFP538
CDIKTVYPAKK.. PVKMPGYHYVDTKLVIRSNDKEFM. KVEEHEIAVARHHPLQSQ					dsFP483
--FE-I-KPN- V----D--F--HYIE-T-QQNYYN V--LT-V-E--YSS-EKIGSKA					dgFP512
VEF-SI-M---. . . --QL--Y--S--D-T-HNEDYT. I--QY-RTEG---LFL					drFP583
VEF-SI-MV-- PS-QL--Y--S--DMT-HNEDYT V--QY-KTQ----FIKPLQ					dmFP592
CHLHTTYRSKKPASALKMPGFHFEDHRIEIMEEVEKGK. CYKQYEAAVGRYCDAAPSKLGHN					asFP600
CQFHTSYKTKK... PVTMPPNHVVEHRIARTDLDKGGN. SVQLTEHAVAHTSVFPF					amFP486
CDFKSIYKAKK... VVKLPDYHFVDHRIEILNHDKDYN. KVTLYENAVARYSLLPSQA					cFP484

FIG. 2A

»

MKCKFVFCLSFLVLAITNANIFLRNEADLEEKTLRIP

FIG. 2B
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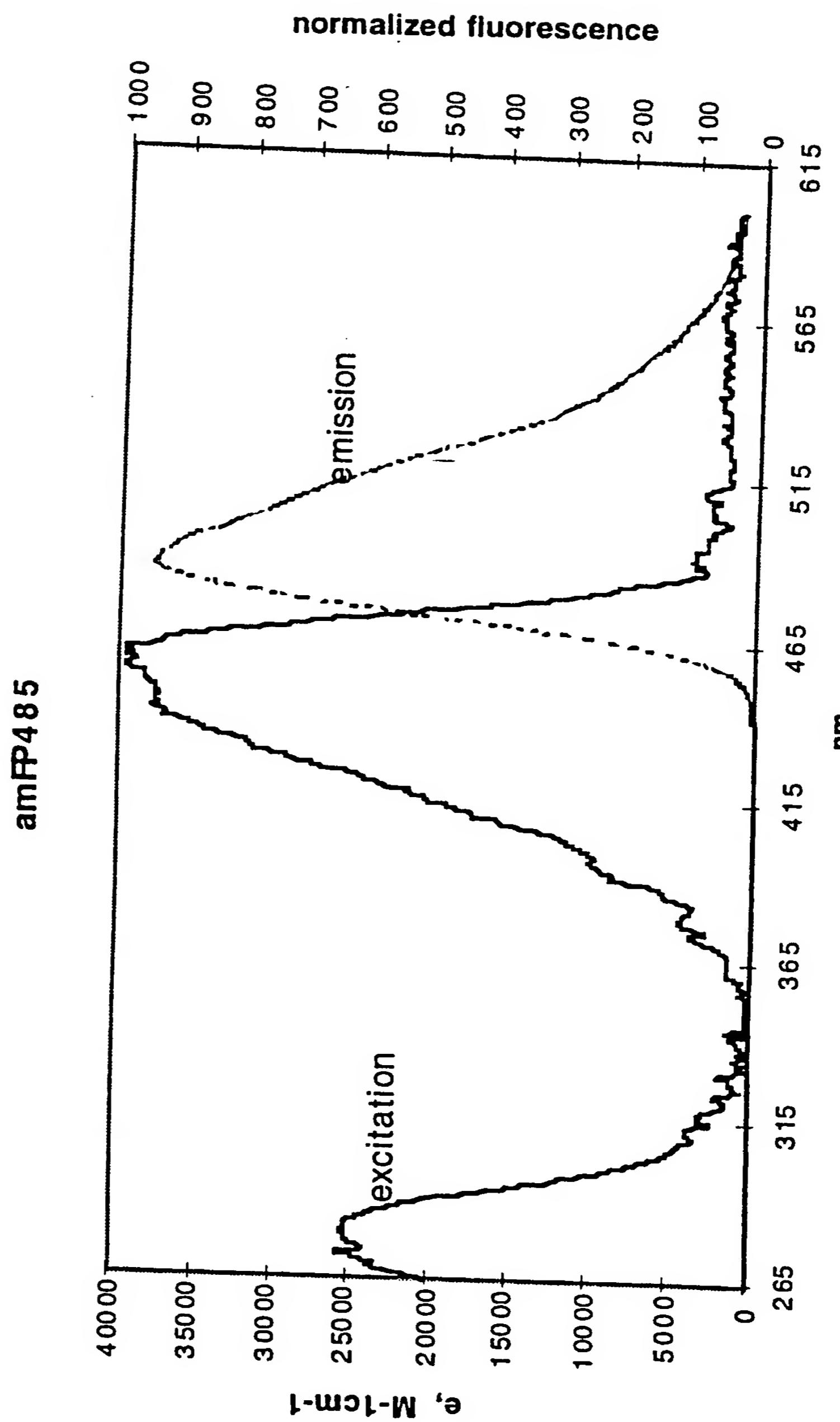


FIG. 3



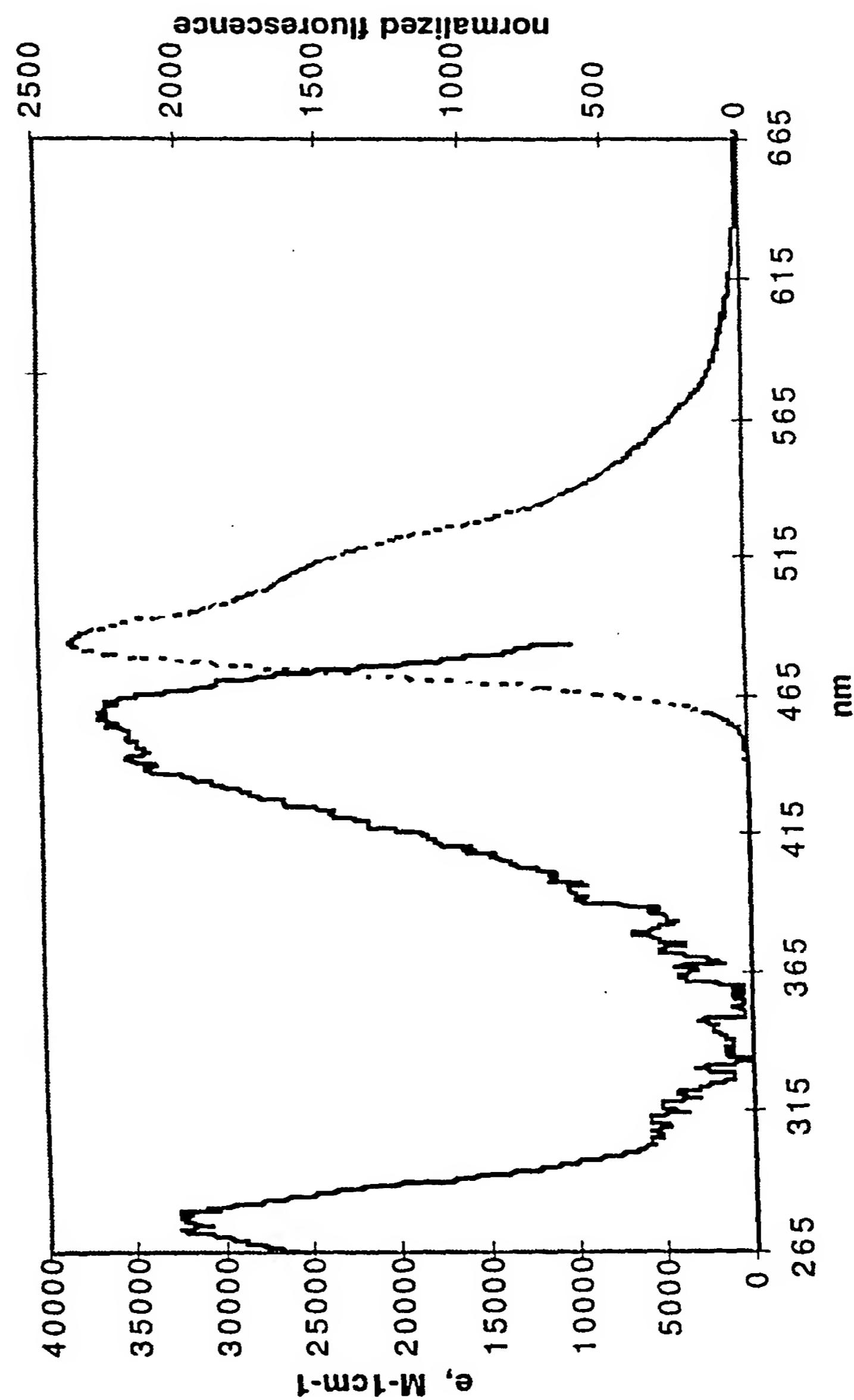
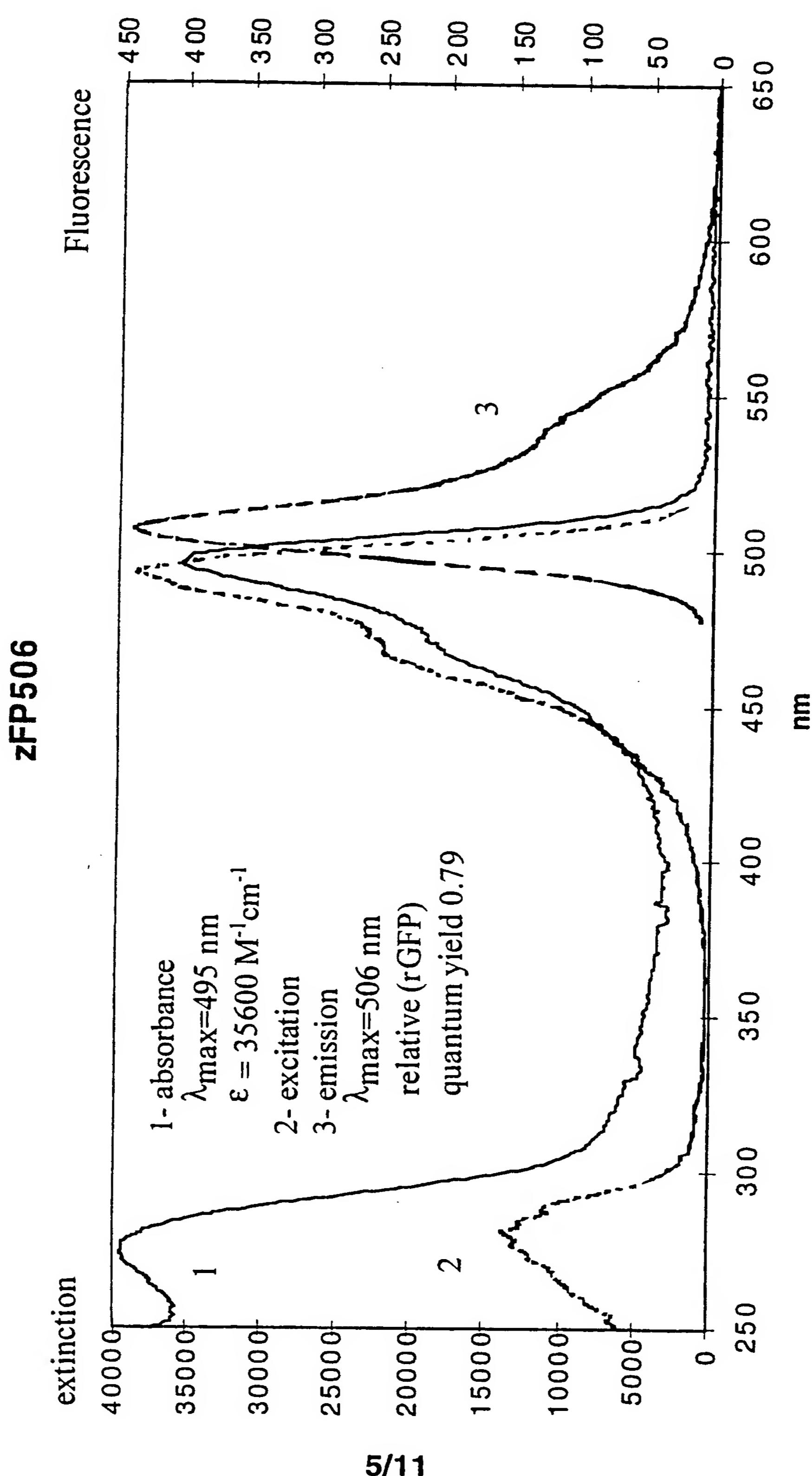


FIG. 4







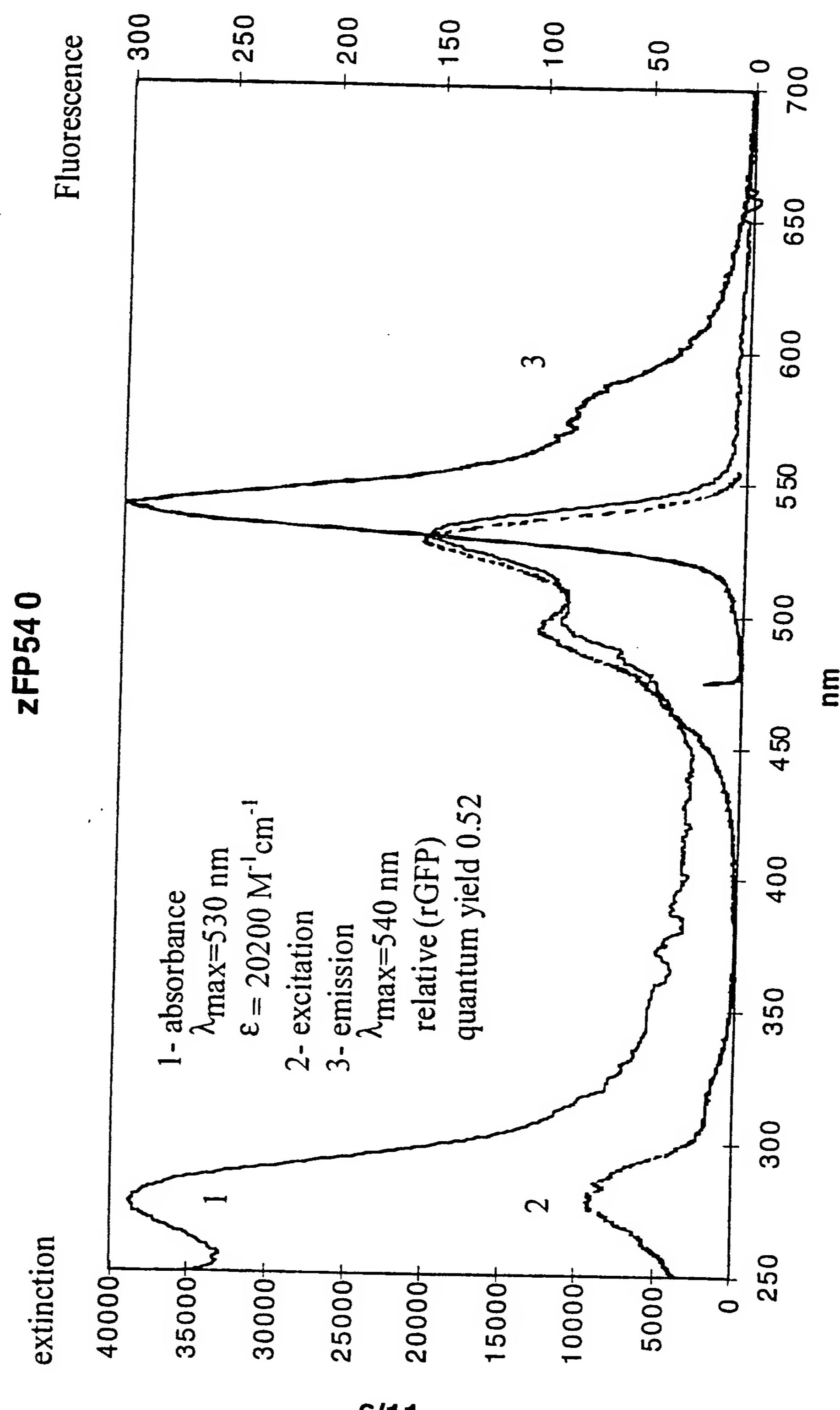
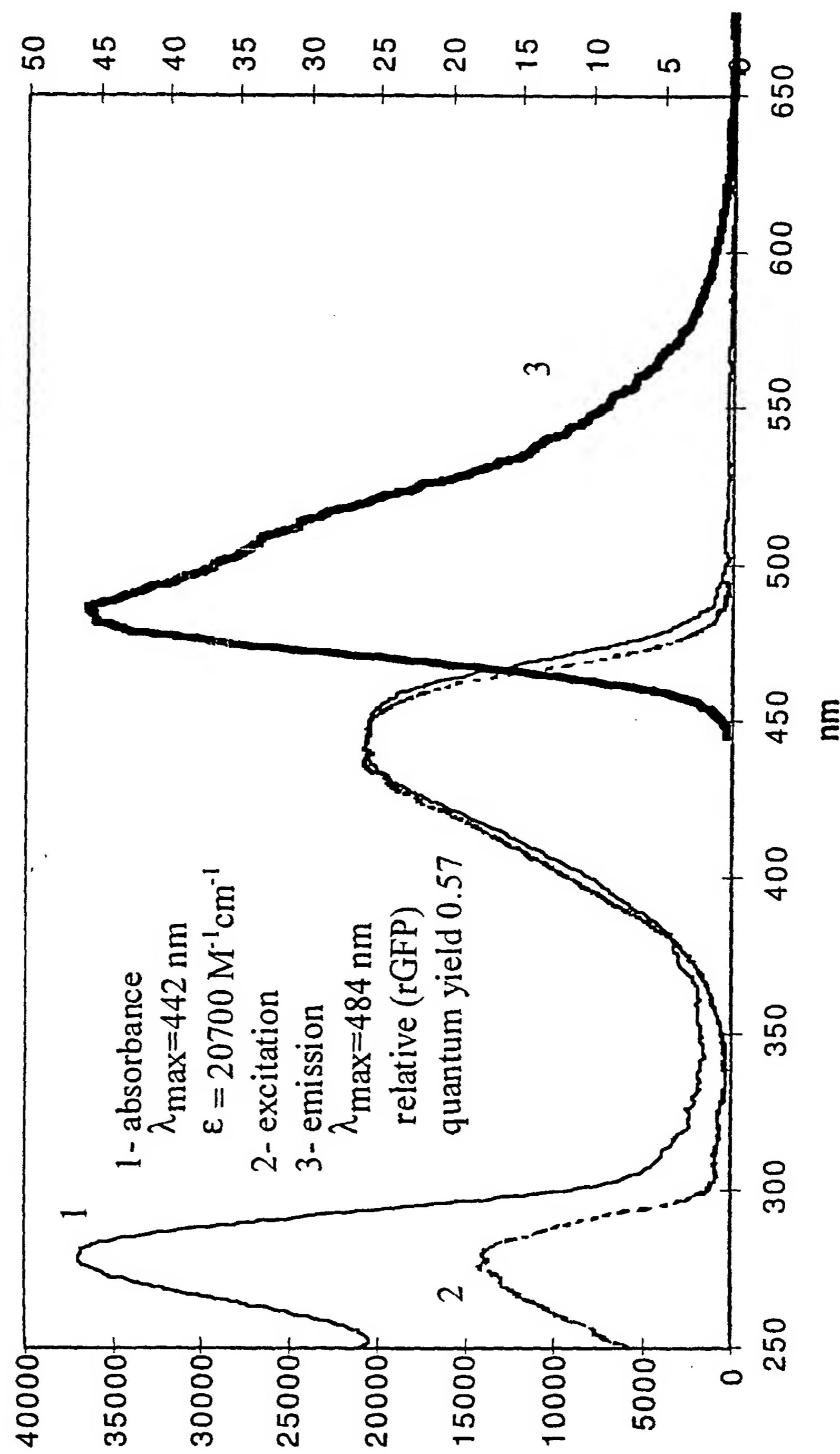


FIG. 6

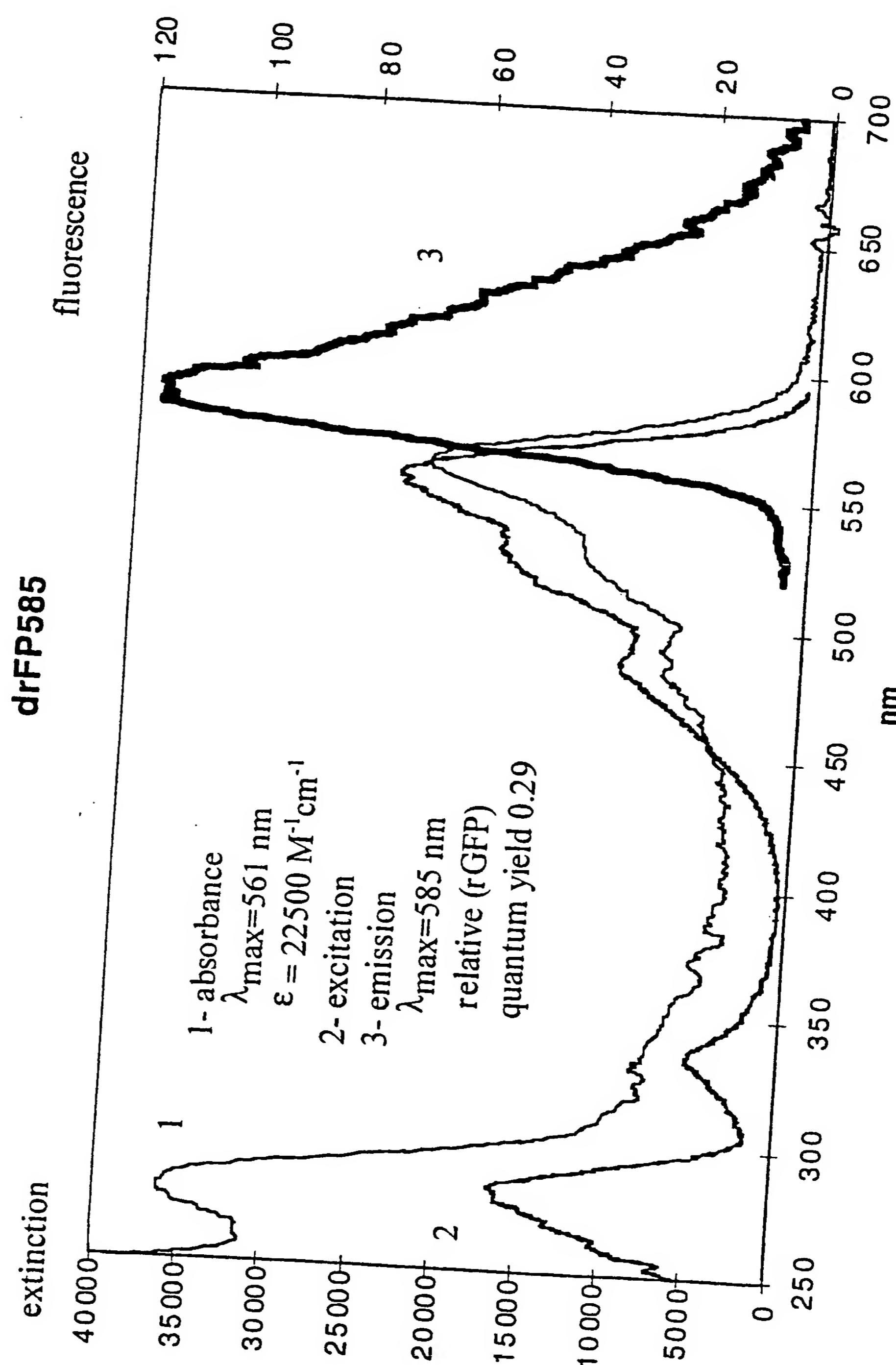
dsFP484

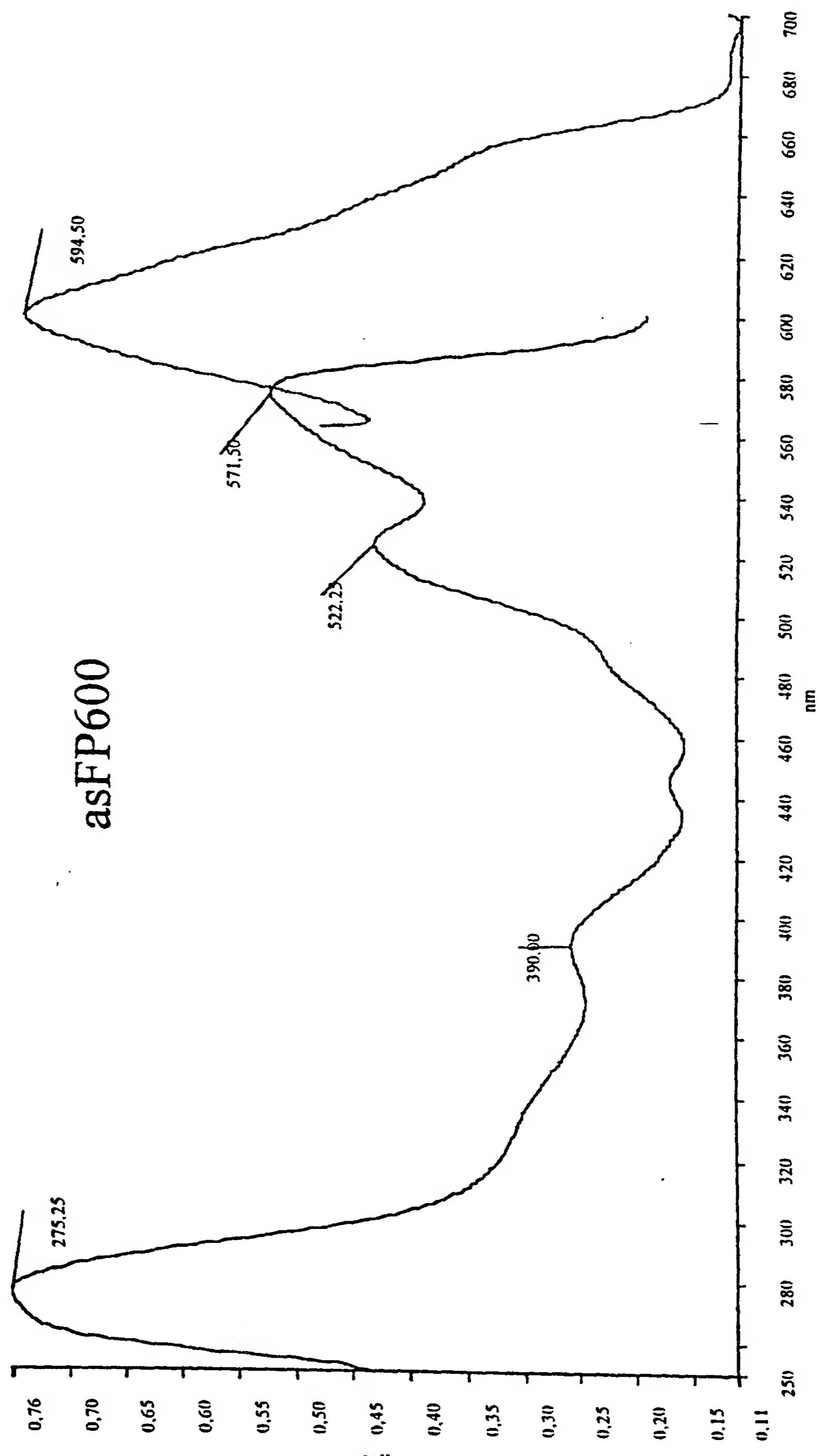
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FIG. 7







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FIG. 9

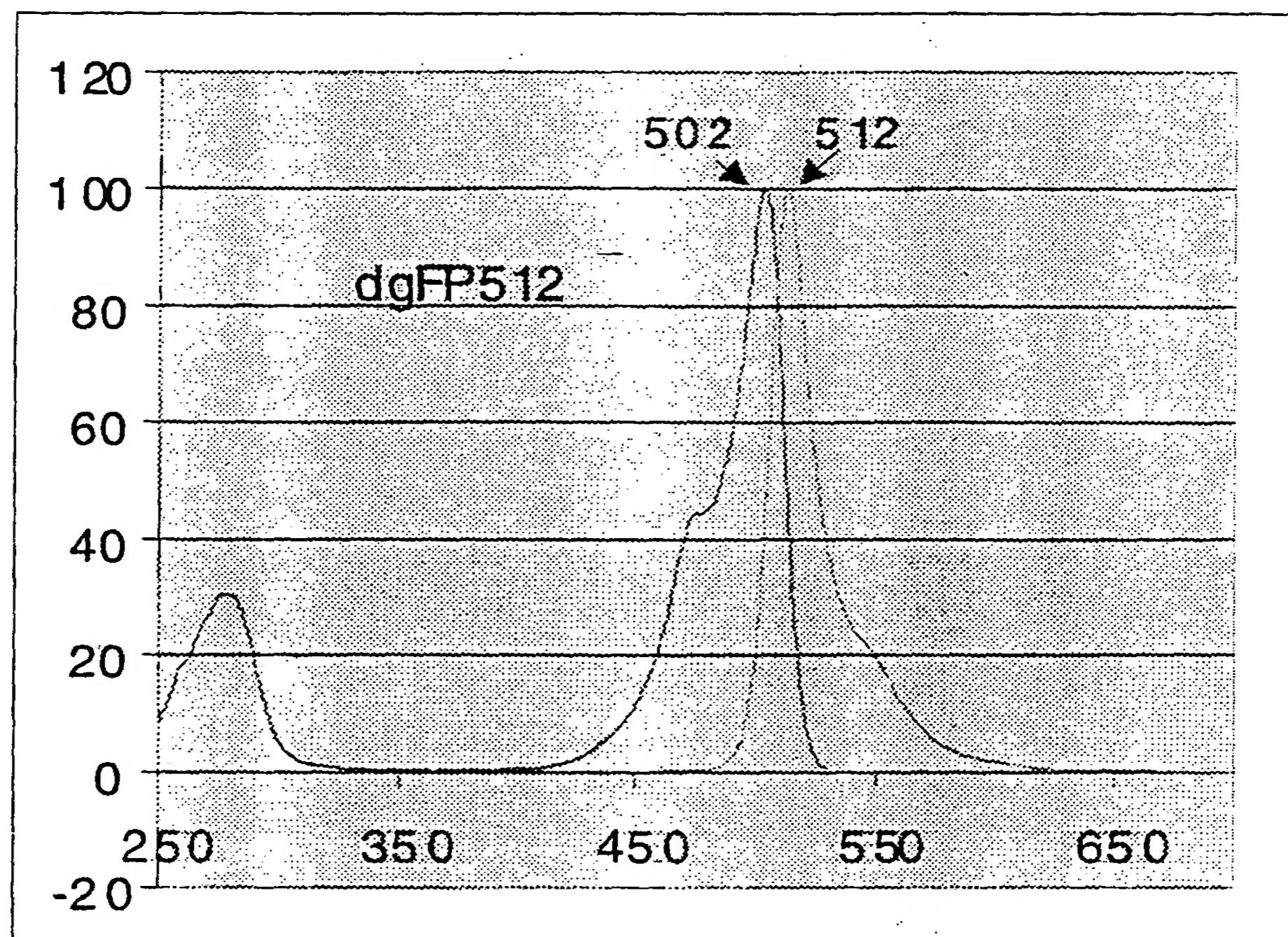


Fig. 10

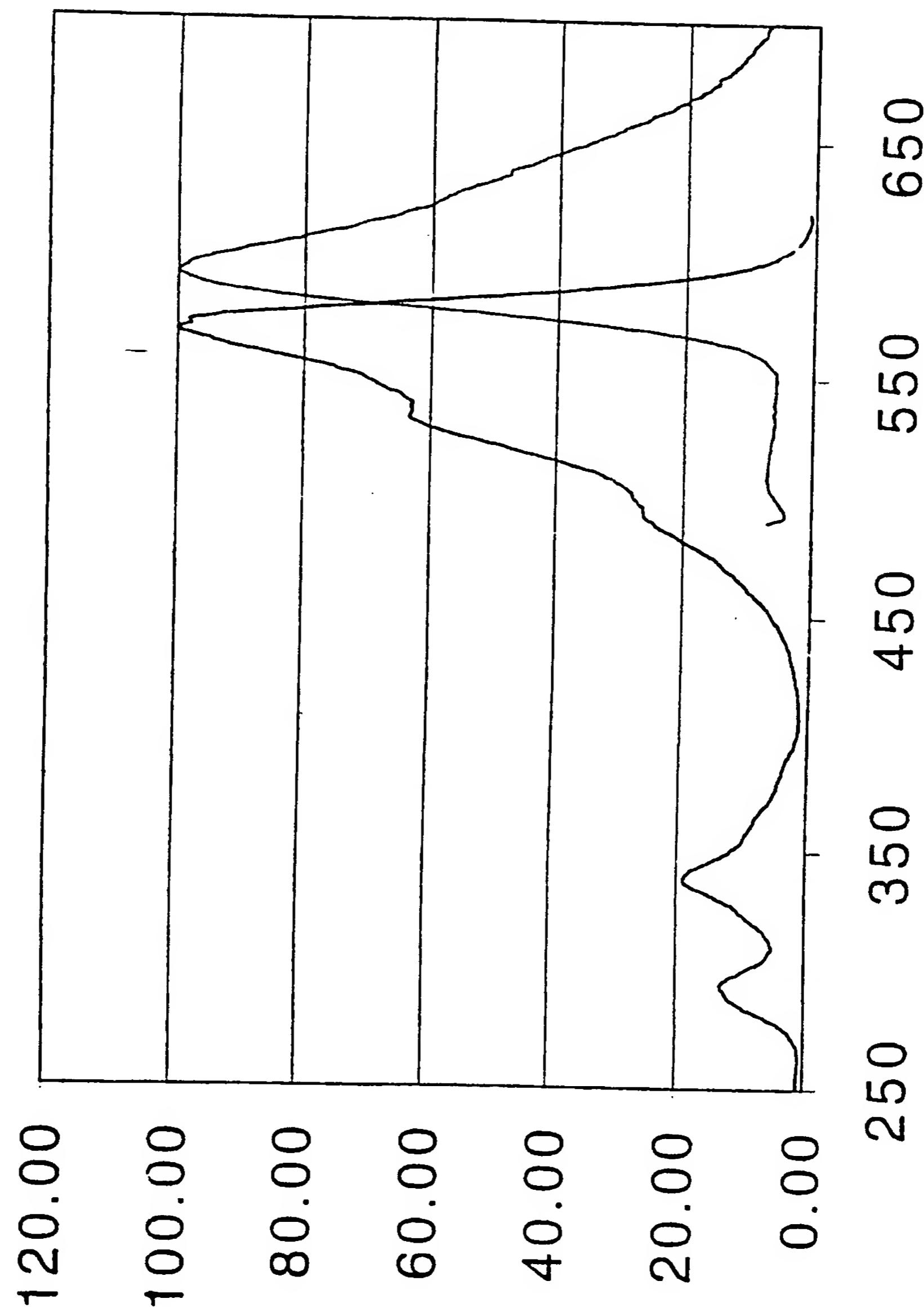


FIG. 11

SEQUENCE LISTING

5 <110> Lukyanov, Sergey A.
Labas, Yulii A.
Matz, Mikhail V.
Fradkov, Arcady F.

10 <120> Fluorescent proteins from non-bioluminescent
species of Class Anthozoa, genes encoding such
proteins and uses thereof

15 <130> D6196PCT
<141> 1999-12-10
<150> 09/210,330
<151> 1998-12-11
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<213> artificial sequence
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<223> primer TS used in cDNA synthesis and RACE
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35 aagcagtggatcaacgcag agt 23

35 <210> 3
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<212> PRT



5 <213> *Aequorea victoria*
 <220>
 <222> 21
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 primer NGH is based; Xaa at position 21
 represents unknown
 <400> 3

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 5
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 <223> primer NGH used for isolation of fluorescent
 protein; n at position 12 represents any of the
 20 four bases
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 gayggctgcg tnaayggdca 20

 25 <210> 5
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 5
 35 <210> 6
 <211> 20



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 protein
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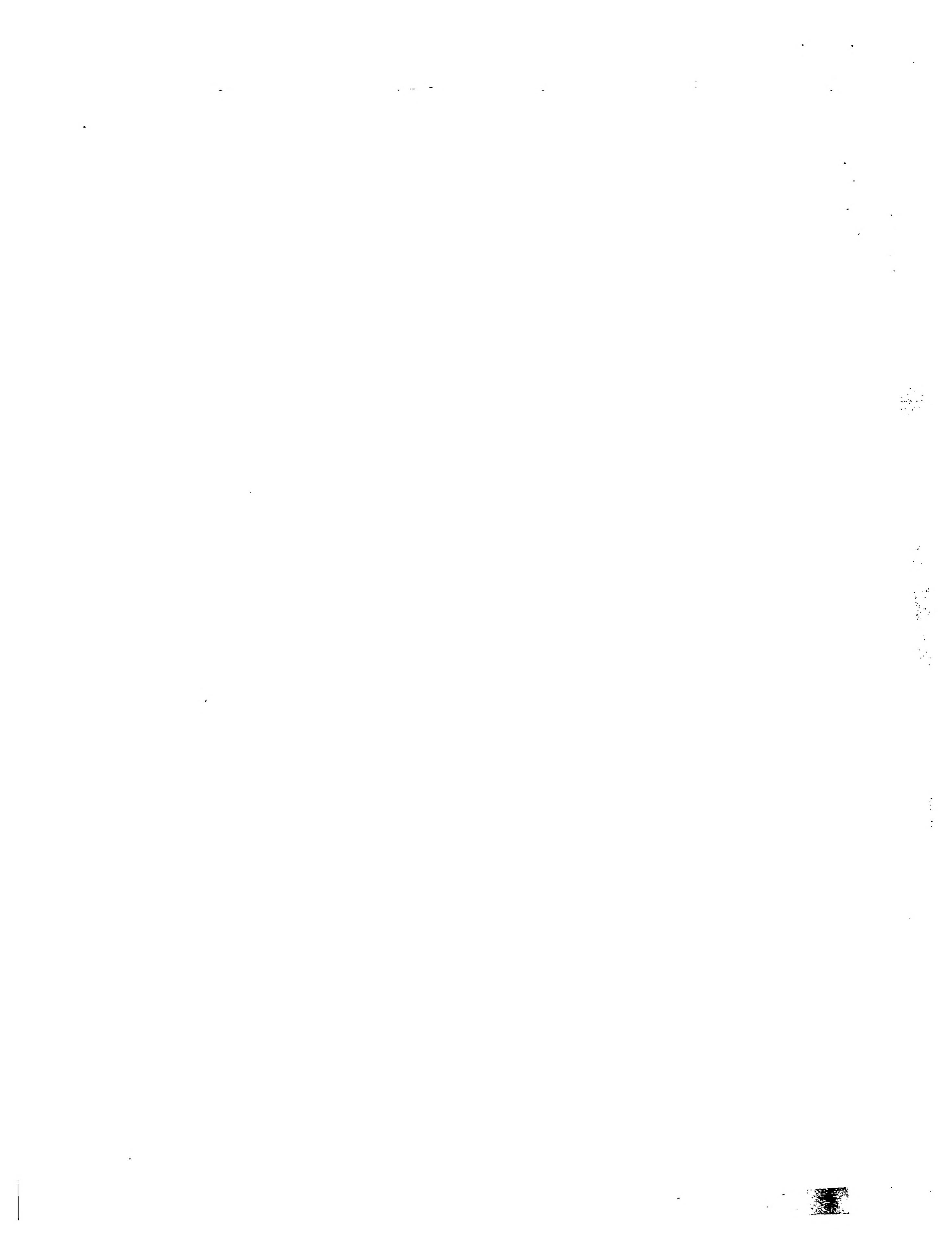
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 5

35 <210> 9
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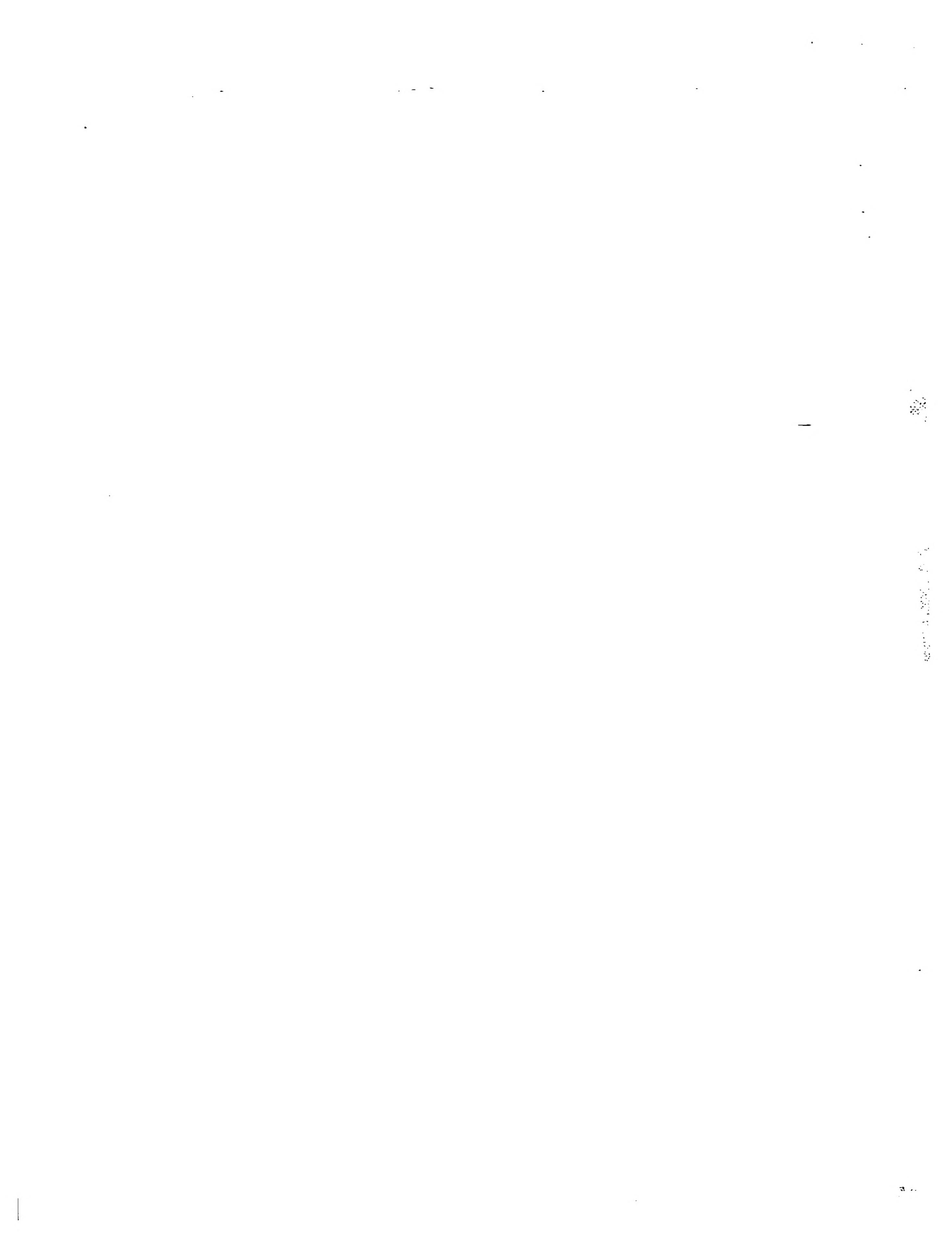
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 <220>
10 <221> primer_bind
 <223> primer NFP used for isolation of fluorescent
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15

14
4
PRT
Aequorea victoria

20 <220>
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25 Gly Pro Val Met

15
21
30 <212> DNA
 <213> artificial sequence
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 <221> primer_bind
 <222> 15

35 <223> primer PVMa used for isolation of fluorescent
 protein; n at position 15 represents any of the



```

        four bases
<400>      15

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5      <210>      16
      <211>      21
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      <220>
10     <221>      primer_bind
      <222>      15
      <223>      primer PVMb used for isolation of fluorescent
                  protein; n at position 15 represents any of the
                  four bases
15     <400>      16

cctgccrayg gtccngtkat g          21

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      <213>      artificial sequence
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      <220>
      <221>      primer_bind
      <223>      primer T7-TS used in cDNA synthesis and RACE
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30 <400>      21

gtcaggcata ctggtaggat          20

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21

10 <210> 23
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19

20 <210> 24
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 25 <220>
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 <223> gene-specific primer used for 5'-RACE for
 Zoanthus sp.
 <400> 24

30 gttcttgaaa tagtctacta tgt

23

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10	<210>	26
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15	<400>	26
	caagcaaatg gcaaaggc	19
20	<210>	27
	<211>	19
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	<223>	gene-specific primer used for 5'-RACE for <i>Discosoma sp. "red"</i>
25	<400>	27
	cggatttgtg gccttcgta	19
30	<210>	28
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	<212>	DNA
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35		



Discosoma striata

	<400>	28
	ttgtcttctt ctgcacaac	19
5	<210>	29
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	<212>	DNA
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10	<221>	primer_bind
	<223>	gene-specific primer used for 5'-RACE for <i>Discosoma striata</i>
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	ctgcacaacg ggtccat	17
15	<210>	30
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	<223>	gene-specific primer used for 5'-RACE for <i>Anemonia sulcata</i>
	<400>	30
25	cctctatctt catttcctgc	20
	<210>	31
	<211>	20
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	<223>	gene-specific primer used for 5'-RACE for <i>Anemonia sulcata</i>
35	<400>	31
	tatcttcatt tcctgcgtac	20

5 <210> 32
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 5 <213> artificial sequence
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 Discosoma sp. "magenta"
10 <400> 32

ttcagcaccc catcacgag 19

15 <210> 33
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 Discosoma sp. "magenta"
20 <400> 33

acgctcagag ctgggttcc 19

25 <210> 34
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 30 <223> gene-specific primer used for 5'-RACE for
 Discosoma sp. "green"
 <400> 34

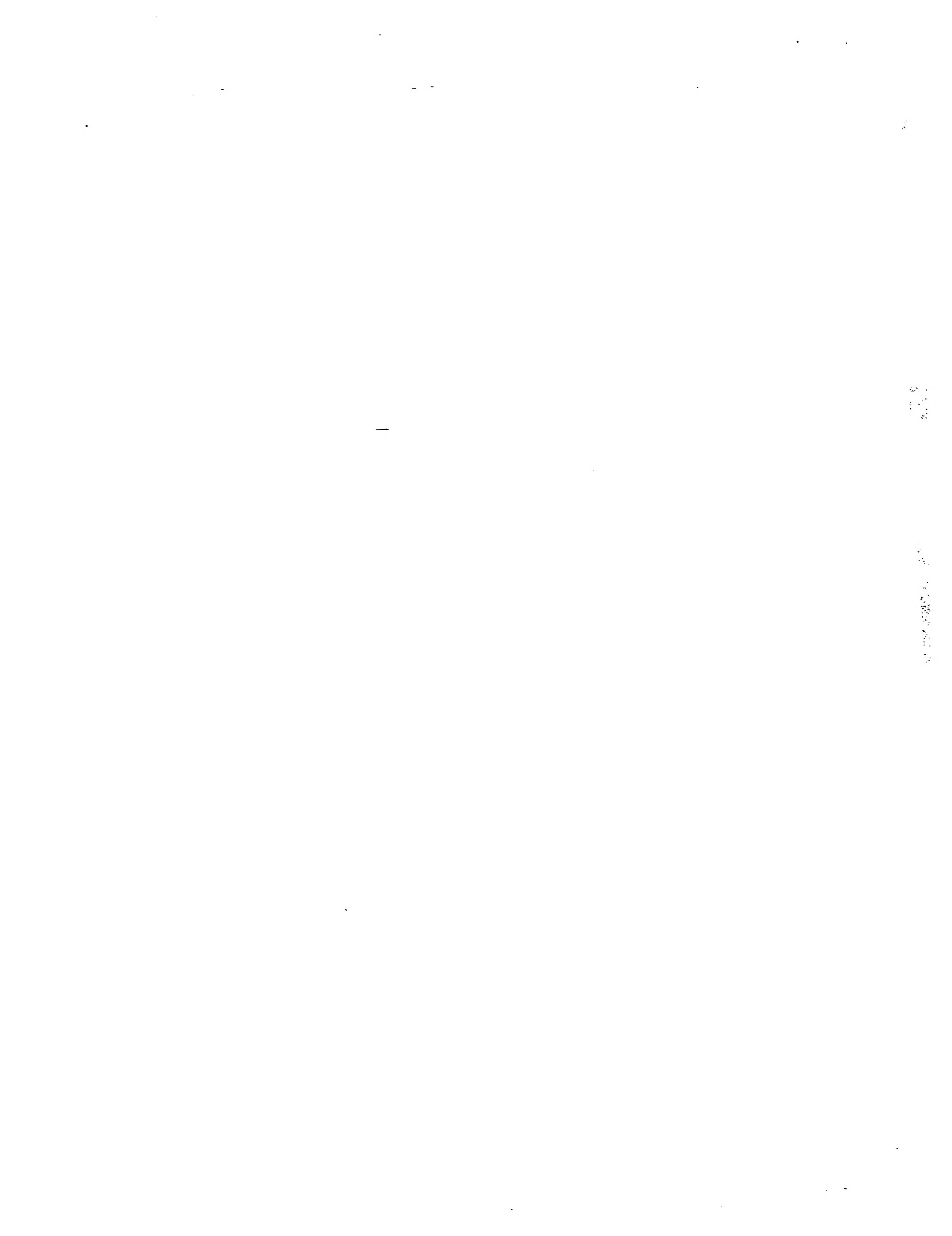
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		<i>Discosoma</i> sp. "green"	
	<400>	35	
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15	<223>	upstream primer used to obtain full coding region	
		of nFPs from <i>Anemonia majano</i>	
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	acatggatcc gctctttcaa acaagtttat c		31
20	<210>	37	
	<211>	34	
	<212>	DNA	
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	<220>		
25	<221>	primer_bind	
	<223>	downstream primer used to obtain full coding	
		region of nFPs from <i>Anemonia majano</i>	
	<400>	37	
	tagtactcga gcttattcgt atttcagtga aatc		34
30	<210>	38	
	<211>	29	
	<212>	DNA	
	<213>	artificial sequence	
	<220>		
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35	<223>	upstream primer used to obtain full coding region	

		of nFPs from <i>Clavularia</i> sp.
<400>	38	
acatggatcc aacatTTTT tgagaaaacg		29
5 <210>	39	
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<223>	upstream primer used to obtain full coding region	
	of nFPs from <i>Clavularia</i> sp.	
<400>	39	—
15 acatggatcc aaagctctaa ccaccatg		28
<210>	40	
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20 <220>		
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	region of nFPs from <i>Clavularia</i> sp.	
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25 tagtactcga gcaacacaaa ccctcagaca a		31
<210>	41	
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30 <213>	artificial sequence	
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	of nFPs from <i>Zoanthus</i> sp.	
35 <400>	41	
acatqqatcc qctcaqtcaa aqcacqqt		28



5	<210>	42
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10	<400>	42
	tagtactcgatgttggaaactacattttatca	32
15	<210>	43
	<211>	31
	<212>	DNA
	<213>	artificial sequence
	<220>	
	<221>	primer_bind
	<223>	upstream primer used to obtain full coding region of nFPs from <i>Discosoma</i> sp. "red"
20	<400>	43
	acatggatccaggcttccaaagatgttatc	31
25	<210>	44
	<211>	29
	<212>	DNA
	<213>	artificial sequence
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	<221>	primer_bind
	<223>	downstream primer used to obtain full coding region of nFPs from <i>Discosoma</i> sp. "red"
30	<400>	44
	tagtactcgaggccaaatgttcagccat	29
35	<210>	45
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		of nFPs from <i>Discosoma striata</i>	
	<400>	45	
	acatggatcc agttggtcca agagtg		28
10	<210>	46	
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15	<223>	downstream primer used to obtain full coding	
		region of nFPs from <i>Discosoma striata</i>	
	<400>	46	
	tagcgagctc tatcatgcct cgtcac		28
20	<210>	47	
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		of nFPs from <i>Anemonia sulcata</i>	
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30	<210>	48	
	<211>	28	
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35	<223>	downstream primer used to obtain full coding	



region of nFPs from *Anemonia sulcata*

<400> 48

tagtactcga gtccttggga gcggcttg 28

5 <210> 49

<211> 30

<212> DNA

<213> artificial sequence

<220>

10 <221> primer_bind

<223> upstream primer used to obtain full coding region of nFPs from *Discosoma sp.* "magenta"

<400> 49

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15 <210> 50

<211> 26

<212> DNA

<213> artificial sequence

20 <220>

<221> primer_bind

<223> downstream primer used to obtain full coding region of nFPs from *Discosoma sp.* "magenta"

<400> 50

25 tagtactcga ggccattacg ctaatc 26

<210> 51

<211> 31

<212> DNA

30 <213> artificial sequence

<220>

<221> primer_bind

<223> upstream primer used to obtain full coding region of nFPs from *Discosoma sp.* "green"

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5	<210>	52
	<211>	29
	<212>	DNA
	<213>	artificial sequence
	<220>	
	<221>	primer_bind
	<223>	downstream primer used to obtain full coding region of nFPs from <i>Discosoma</i> sp. "green"
10	<400>	52
	tagtactcg a gattcggtt a atgccttg	29
	<210>	53
	<211>	33
15	<212>	DNA
	<213>	artificial sequence
	<220>	
	<221>	primer_bind
	<223>	TS-oligo used in cDNA synthesis and RACE
20	<400>	53
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	<210>	54
	<211>	238
25	<212>	PRT
	<213>	<i>Aequorea victoria</i>
	<220>	
	<223>	amino acid sequence of GFP
	<400>	54
30	Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
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	15	
	Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser	
	20	25
	30	
	Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys	
35	35	40
	45	
	Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu	
	50	55
	60	

Val	Thr	Thr	Phe	Ser	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	
							65			70				75	
Asp	His	Met	Lys	Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	
							80			85				90	
5	Gly	Tyr	Val	Gln	Glu	Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn
								95			100				105
Tyr	Lys	Thr	Arg	Ala	Glu	Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	
							110			115				120	
Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	
10							125			130				135	
Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn	Tyr	Asn	Ser	His	Asn	Val	
							140			145				150	
Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	Gly	Ile	Lys	Val	Asn	Phe	
							155			160				165	
15	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	Val	Gln	Leu	Ala	Asp
							170			175				180	
His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	Pro	Val	Leu	Leu	
							185			190				195	
Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser	Lys	Asp	
20							200			205				210	
Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val	Thr	
							215			220				225	
Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Lys			
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35	Tyr	His	Met	Asp	Gly	Cys	Val	Asn	Gly	His	Tyr	Phe	Thr	Val	
							20			25				30	
	Gly	Glu	Gly	Asn	Gly	Lys	Pro	Tyr	Glu	Gly	Thr	Gln	Thr	Ser	
							35			40				45	



Phe	Lys	Val	Thr	Met	Ala	Asn	Gly	Gly	Pro	Leu	Ala	Phe	Ser	Phe	
				50					55					60	
Asp	Ile	Leu	Ser	Thr	Val	Phe	Lys	Tyr	Gly	Asn	Arg	Cys	Phe	Thr	
				65				70						75	
5	Ala	Tyr	Pro	Thr	Ser	Met	Pro	Asp	Tyr	Phe	Lys	Gln	Ala	Phe	Pro
				80					85					90	
Asp	Gly	Met	Ser	Tyr	Glu	Arg	Thr	Phe	Thr	Tyr	Glu	Asp	Gly	Gly	
				95					100					105	
10	Val	Ala	Thr	Ala	Ser	Trp	Glu	Ile	Ser	Leu	Lys	Gly	Asn	Cys	Phe
				110					115					120	
Glu	His	Lys	Ser	Thr	Phe	His	Gly	Val	Asn	Phe	Pro	Ala	Asp	Gly	
				125					130					135	
15	Pro	Val	Met	Ala	Lys	Lys	Thr	Thr	Gly	Trp	Asp	<u>Pro</u>	Ser	Phe	Glu
				140					145					150	
20	Lys	Met	Thr	Val	Cys	Asp	Gly	Ile	Leu	Lys	Gly	Asp	Val	Thr	Ala
				155					160					165	
25	Phe	Leu	Met	Leu	Gln	Gly	Gly	Asn	Tyr	Arg	Cys	Gln	Phe	His	
				170					175					180	
30	Thr	Ser	Tyr	Lys	Thr	Lys	Lys	Pro	Val	Thr	Met	Pro	Pro	Asn	His
				185					190					195	
35	Val	Val	Glu	His	Arg	Ile	Ala	Arg	Thr	Asp	Leu	Asp	Lys	Gly	Gly
				200					205					210	
40	Asn	Ser	Val	Gln	Leu	Thr	Glu	His	Ala	Val	Ala	His	Ile	Thr	Ser
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45	Val	Val	Pro	Phe											

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	<211>	266													
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	<213>	<i>Clavularia</i> sp.													
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	Ile	Thr	Asn	Ala	Asn	Ile	Phe	Leu	Arg	Asn	Glu	Ala	Asp	Phe	Glu

	20	25	30
	Glu Lys Thr Phe Arg Ile Pro Lys Ala Leu Thr Thr Met Gly Val		
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	Ile Lys Pro Asp Met Lys Ile Lys Leu Lys Met Glu Gly Asn Val		
5	50	55	60
	Asn Gly His Ala Phe Val Ile Glu Gly Glu Gly Glu Lys Pro		
	65	70	75
	Tyr Asp Gly Thr His Thr Leu Asn Leu Glu Val Lys Glu Gly Ala		
	80	85	90
10	Pro Leu Pro Phe Ser Tyr Asp Ile Leu Ser Asn Ala Phe Gln Tyr		
	95	100	105
	Gly Asn Arg Ala Leu Thr Lys Tyr Pro Asp Asp Ile Ala Asp Tyr		
	110	115	120
	Phe Lys Gln Ser Phe Pro Glu Gly Tyr Ser Trp Glu Arg Thr Met		
15	125	130	135
	Thr Phe Glu Asp Lys Gly Ile Val Lys Val Lys Ser Asp Ile Ser		
	140	145	150
	Met Glu Glu Asp Ser Phe Ile Tyr Glu Ile Arg Phe Asp Gly Met		
	155	160	165
20	Asp Phe Pro Pro Asn Gly Pro Val Met Gln Lys Lys Thr Leu Lys		
	170	175	180
	Trp Glu Pro Ser Thr Glu Ile Met Tyr Val Arg Asp Gly Val Leu		
	185	190	195
	Val Gly Asp Ile Ser His Ser Leu Leu Leu Glu Gly Gly His		
25	200	205	210
	Tyr Arg Cys Asp Phe Lys Ser Ile Tyr Lys Ala Lys Lys Val Val		
	215	220	225
	Lys Leu Pro Asp Tyr His Phe Val Asp His Arg Ile Glu Ile Leu		
	230	235	240
30	Asn His Asp Lys Asp Tyr Asn Lys Val Thr Leu Tyr Glu Asn Ala		
	245	250	255
	Val Ala Arg Tyr Ser Leu Leu Pro Ser Gln Ala		
	260	265	
35	<210>	57	
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<213> *Zoanthus sp.*
<220>
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<400> 57

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Arg Met Glu Gly Cys Val Asp Gly His Lys Phe Val Ile Thr Gly
20 25 30
Glu Gly Ile Gly Tyr Pro Phe Lys Gly Lys Gln Ala Ile Asn Leu
10 35 40 45
Cys Val Val Glu Gly Gly Pro Leu Pro Phe Ala Glu Asp Ile Leu
50 55 60
Ser Ala Ala Phe Asn Tyr Gly Asn Arg Val Phe Thr Glu Tyr Pro
65 70 75
15 Gln Asp Ile Val Asp Tyr Phe Lys Asn Ser Cys Pro Ala Gly Tyr
80 85 90
Thr Trp Asp Arg Ser Phe Leu Phe Glu Asp Gly Ala Val Cys Ile
95 100 105
Cys Asn Ala Asp Ile Thr Val Ser Val Glu Glu Asn Cys Met Tyr
20 110 115 120
His Glu Ser Lys Phe Tyr Gly Val Asn Phe Pro Ala Asp Gly Pro
125 130 135
Val Met Lys Lys Met Thr Asp Asn Trp Glu Pro Ser Cys Glu Lys
140 145 150
25 Ile Ile Pro Val Pro Lys Gln Gly Ile Leu Lys Gly Asp Val Ser
155 160 165
Met Tyr Leu Leu Leu Lys Asp Gly Gly Arg Leu Arg Cys Gln Phe
170 175 180
Asp Thr Val Tyr Lys Ala Lys Ser Val Pro Arg Lys Met Pro Asp
30 185 190 195
Trp His Phe Ile Gln His Lys Leu Thr Arg Glu Asp Arg Ser Asp
200 205 210
Ala Lys Asn Gln Lys Trp His Leu Thr Glu His Ala Ile Ala Ser
215 220 225
35 Gly Ser Ala Leu Pro
230

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5	<213>	<i>Zoanthus sp.</i>	
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	Met Ala His Ser Lys His Gly Leu Lys Glu Glu Met Thr Met Lys		
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	Tyr His Met Glu Gly Cys Val Asn Gly His Lys Phe Val Ile Thr		
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	Gly Glu Gly Ile Gly Tyr Pro Phe Lys Gly Lys Gln Thr Ile Asn		
	35	40	45
15	Leu Cys Val Ile Glu Gly Gly Pro Leu Pro Phe Ser Glu Asp Ile		
	50	55	60
	Leu Ser Ala Gly Phe Lys Tyr Gly Asp Arg Ile Phe Thr Glu Tyr		
	65	70	75
	Pro Gln Asp Ile Val Asp Tyr Phe Lys Asn Ser Cys Pro Ala Gly		
20	80	85	90
	Tyr Thr Trp Gly Ser Phe Leu Phe Glu Asp Gly Ala Val Cys Ile		
	95	100	105
	Cys Asn Val Asp Ile Thr Val Ser Val Lys Glu Asn Cys Ile Tyr		
	110	115	120
25	His Lys Ser Ile Phe Asn Gly Met Asn Phe Pro Ala Asp Gly Pro		
	125	130	135
	Val Met Lys Lys Met Thr Thr Asn Trp Glu Ala Ser Cys Glu Lys		
	140	145	150
	Ile Met Pro Val Pro Lys Gln Gly Ile Leu Lys Gly Asp Val Ser		
30	155	160	165
	Met Tyr Leu Leu Leu Lys Asp Gly Gly Arg Tyr Arg Cys Gln Phe		
	170	175	180
	Asp Thr Val Tyr Lys Ala Lys Ser Val Pro Ser Lys Met Pro Glu		
	185	190	195
35	Trp His Phe Ile Gln His Lys Leu Leu Arg Glu Asp Arg Ser Asp		
	200	205	210
	Ala Lys Asn Gln Lys Trp Gln Leu Thr Glu His Ala Ile Ala Phe		
	215	220	225

Pro Ser Ala Leu Ala

230

5 <210> 59
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 <212> PRT
 <213> *Discosoma striata*
 <220>
 10 <223> amino acid sequence of dsFP483
 <400> 59

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							20							30
Gly	Lys	Gly	Lys	Gly	Gln	Pro	Asn	Glu	Gly	Thr	Asn	Thr	Val	Thr
							35							45
Leu	Glu	Val	Thr	Lys	Gly	Gly	Pro	Leu	Pro	Phe	Gly	Trp	His	Ile
							50							60
20 Leu	Cys	Pro	Gln	Phe	Gln	Tyr	Gly	Asn	Lys	Ala	Phe	Val	His	His
							65							75
Pro	Asp	Asn	Ile	His	Asp	Tyr	Leu	Lys	Leu	Ser	Phe	Pro	Glu	Gly
							80							90
Tyr	Thr	Trp	Glu	Arg	Ser	Met	His	Phe	Glu	Asp	Gly	Gly	Leu	Cys
							95							105
25 Cys	Ile	Thr	Asn	Asp	Ile	Ser	Leu	Thr	Gly	Asn	Cys	Phe	Tyr	Tyr
							110							120
Asp	Ile	Lys	Phe	Thr	Gly	Leu	Asn	Phe	Pro	Pro	Asn	Gly	Pro	Val
							125							135
30 Val	Gln	Lys	Lys	Thr	Thr	Gly	Trp	Glu	Pro	Ser	Thr	Glu	Arg	Leu
							140							150
Tyr	Pro	Arg	Asp	Gly	Val	Leu	Ile	Gly	Asp	Ile	His	His	Ala	Leu
							155							165
35 Thr	Val	Glu	Gly	Gly	Gly	His	Tyr	Ala	Cys	Asp	Ile	Lys	Thr	Val
							170							180
Tyr	Arg	Ala	Lys	Lys	Ala	Ala	Leu	Lys	Met	Pro	Gly	Tyr	His	Tyr
							185							195

Val	Asp	Thr	Lys	Leu	Val	Ile	Trp	Asn	Asn	Asp	Lys	Glu	Phe	Met	
				200				205				210			
Lys	Val	Glu	Glu	His	Glu	Ile	Ala	Val	Ala	Arg	His	His	Pro	Phe	
				215				220				225			
5	Tyr	Glu	Pro	Lys	Lys	Asp	Lys								
				230											
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10	<211>			225											
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					20				25				30		
	Gly	Glu	Gly	Glu	Gly	Arg	Pro	Tyr	Glu	Gly	His	Asn	Thr	Val	Lys
20						35			40				45		
	Leu	Lys	Val	Thr	Lys	Gly	Gly	Pro	Leu	Pro	Phe	Ala	Trp	Asp	Ile
						50			55				60		
	Leu	Ser	Pro	Gln	Phe	Gln	Tyr	Gly	Ser	Lys	Val	Tyr	Val	Lys	His
						65			70				75		
25	Pro	Ala	Asp	Ile	Pro	Asp	Tyr	Lys	Lys	Leu	Ser	Phe	Pro	Glu	Gly
						80			85				90		
	Phe	Lys	Trp	Glu	Arg	Val	Met	Asn	Phe	Glu	Asp	Gly	Gly	Val	Val
						95			100				105		
	Thr	Val	Thr	Gln	Asp	Ser	Ser	Leu	Gln	Asp	Gly	Cys	Phe	Ile	Tyr
30						110			115				120		
	Lys	Val	Lys	Phe	Ile	Gly	Val	Asn	Phe	Pro	Ser	Asp	Gly	Pro	Val
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	Ala Thr Ser Asn Ile Ser Val Val Gly Asp Thr Phe Asn Tyr Asp		
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Phe Lys Trp Glu Arg Val Met Asn Phe Glu Asp Gly Gly Val Val
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Glu Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp Gly Pro Val
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Met Gln Arg Arg Thr Arg Gly Trp Glu Ala Ser Ser Glu Arg Leu
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15 Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp Ile His Met Ala Leu
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Arg Leu Glu Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile
175 180 185

Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly Tyr Tyr Tyr
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Val Asp Ser Lys Leu Asp Met Thr Ser His Asn Glu Asp Tyr Thr
205 210 215

Val Val Glu Gln Tyr Glu Lys Thr Gln Gly Arg His His Pro Phe
220 225 230

25 Ile Lys Pro Leu Gln
235

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/29405

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C07K 14/435
 US CL :435/6, 69.1; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 968; 530/350; 424/9.6, 436/172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
***	The sequence diskette submitted with the description was defective; thus the references listed below were obtained solely by a WORD search, and not by a search of the SEQ ID NOS.	***
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotechnology. October 1999, Volume 17, No. 10, pages 969-973, entire document.	1-10
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999, entire document.	3-10

Further documents are listed in the continuation of Box C.

See patent family annex.

•	Special categories of cited documents:	•T•	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
•A•	document defining the general state of the art which is not considered to be of particular relevance	•X•	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
•E•	earlier document published on or after the international filing date	•Y•	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
•L•	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	•&•	document member of the same patent family
•O•	document referring to an oral disclosure, use, exhibition or other means		
•P•	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

18 FEBRUARY 2000

Date of mailing of the international search report

02 MAR 2000

Name and mailing address of the ISA/US
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